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Dexamethasone regulates expression of BRUCE/Apollon and the proliferation of neural progenitor cells

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ABSTRACT

Glucocorticoid hormones (GHs) regulate cell proliferation of neural progenitor cells (NPCs) contributing to reduction of neurogenesis after stress. We show here that dexamethasone (Dex) decreases BRUCE/Apollon (BRUCE) in cultured NPCs in a GH-receptor-dependent manner. Downregulation of BRUCE by Dex or using silencing RNA reduced the number of proliferating NPCs, whilst overexpression of BRUCE counteracted the effect of Dex. Dex also elevated the deubiquitinating enzyme, Usp8/Ubpy, which via Nrdp1 decreases BRUCE. The results show that BRUCE is a target for GHs in the NPCs, and that BRUCE controls cell division of NPCs and possibly of other stem cells.

Structured summary:
MINT-7148564: *Nrdp1* (uniprotkb:Q8BH75) *physically interacts* (MI:0914) with *BRUCE* (uniprotkb:O88738) by *anti bait co-immunoprecipitation* (MI:0006)
MINT-7148555: *Nrdp1* (uniprotkb:Q8BH75) *physically interacts* (MI:0914) with *Usp8* (uniprotkb:Q80U87) by *anti bait co-immunoprecipitation* (MI:0006)

1. Introduction

Neural progenitor cells (NPCs) are highly dividing cells with self-renewal capacity that give rise to both neurons and glial cells. These cells are present in the ventricular neuroepithelium of developing brain, and in discrete neurogenic areas in the adult brain [1,2]. NPCs are regulated by intrinsic and extrinsic factors that influence neurogenesis and brain tissue homeostasis [1,2]. We have previously reported that high levels of glucocorticoid hormones (GHs) inhibit proliferation of NPCs, and that levels of the cell cycle regulator cyclinD1 are changed [3]. Part of this regulation involves protein ubiquitination, but the precise mechanisms are not fully understood.

In this study, we observed that the synthetic GH dexamethasone (Dex) regulates cell proliferation via reducing levels of the protein BRUCE in the NPCs. BRUCE belongs to the inhibitor of apoptosis proteins (IAPs) family [4,5], and has a molecular mass of about 530 kDa [6–8]. Mice with gene deletion of BRUCE die in utero indicating an important role for BRUCE during early mouse development [9,10]. However, the causes for prenatal death of mice lacking BRUCE are unclear, and the protein may regulate functions other than cell death [9–11]. We have recently shown that BRUCE is expressed in the nervous system, and that the protein levels are decreased in neurons following an excitotoxic brain injury [12]. In the present work, we observed that NPCs express BRUCE and that the protein is a target for the action of GH in NPCs, suggesting an important role of BRUCE in stem cell proliferation and neurogenesis.

2. Materials and methods

2.1. BrdU labeling and immunohistochemistry

BrdU labeling of dividing NPCs in the neuroepithelium in embryonic rats was as described before [3]. Brain sections were double immunostained using antibodies against BRUCE (1:300; BD Biosciences) and Ki67 (1:100; BD Biosciences).

Abbreviations: AS, antisense; BIR, baculovirus inhibitory repeat; BRUCE/Apollon, baculoviral inhibitor of apoptosis repeat-containing 6 gene; Dex, dexamethasone; E, embryonic day; EGF, epidermal growth factor; GHs, glucocorticoid hormones; IAP, inhibitor of apoptosis protein; NPCs, neural progenitor cells; siRNA, silencing RNA

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2.2. NPC cultures

NPCs were prepared from embryonic (E) 17 old rat lateral ventricular wall and cultured in the presence of 20 ng/ml of epidermal growth factor (EGF; Preprotech) as described before [3,13,14]. Immunostaining using the Ki67 antibody was performed to estimate the number of proliferating cells [13,14]. To induce cell differentiation, NPCs were cultured for 5 days without EGF on poly-DL-ornithine coated dishes. Neurons and astrocytes were identified by immunostaining using anti-Tau and anti-GFAP antibodies, respectively [3]. NPCs were also double stained for BRUCE (1:300), and the β-coatomer-protein (β-COP; 1:500; Abcam) a marker for Golgi and vesicle membranes.

2.3. Cell viability

Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, MTT (0.5 mg/ml, Sigma) assay as described [3,13], or by counting the number of apoptotic/condensed nuclei stained by 4 μg/ml Hoechst 33342 in four different fields per well and using a Zeiss fluorescence microscopy [3,15]. Statistical analyses were done by Student's *t*-test.

2.4. Overexpression and downregulation experiments

Plasmids encoding BRUCE [7] and Usp8/Ubpy [16] were transfected into NPCs using the Amaxa Nucleofector system [3], and cell clones were selected for with 250 μg/ml G418 before experiments. To downregulate BRUCE, we employed phosphorothiolated oligonucleotides corresponding to nucleotides 948–967 and 1046–1064 (Interactiva Co Germany) in the rat sequence. 2×10^6 NPCs were transfected with a mixture of 2–4 μM antisense (AS) oligonucleotides using the Amaxa Nucleofector system and non-scrambled oligonucleotides were used as control. Equal number of control and treated cells were incubated for 72 h followed by cell counting and the MTT assay. Staurosporine (25 nM) was added to half of the cultures to induce cell death. Silencing RNA (siRNA) constructs against BRUCE or cyclinD1 were purchased from Ambion Inc., and 100 nM siRNA was transfected as above using 5×10^6 NPCs.

RNA isolation, cDNA synthesis and semiquantitative PCR were done using 30 cycles essentially as described before [3]. For BRUCE the following primers were used: upstream primer, 5′-CTCTTACTCCGAGCGAT-3′; downstream primer, 5′-CTTAACAGGGGGAGGC-3′. Annealing was for 30 s at 54 °C, and β-actin was run as control.

2.5. Immunoblotting and immunoprecipitation

Lysates of NPCs were subjected to SDS–PAGE followed by immunoblotting using antibodies against BRUCE (1:500), Usp8/Ubpy (1:300; Abcam), Nrdp1 (1:4000; Bethyl Laboratories), α-spectrin (1:1000; Chemicon), α-actin (1:5000, Sigma). Secondary peroxidase-conjugated antibodies (1:2500, Jackson Laboratories) were added for 2 h and detection was performed using SuperSignal West Pico Substrate (Pierce). Quantification was performed using GelDoc (Bio-Rad). Immunoprecipitation of Nrdp1 from lysates of control and Dex treated NPCs was performed using protein A-Sepharose essentially as described before [3] followed by immunoblotting for Usp8, BRUCE and Nrdp1.

3. Results

3.1. Embryonic NPCs express BRUCE

BrdU labeling of dividing cells in embryonic neuroepithelium combined with immunostaining showed the presence of BRUCE in proliferating NPCs (Fig. 1A). NPCs cultivated as neurospheres expressed BRUCE (Fig. 1B), and immunostaining localized the protein to membranes structures including the Golgi network as revealed by the marker, β-COP (Fig. 1C). Removal of EGF from the culture medium induces cell differentiation and decreased the levels of BRUCE (Fig. 1D). Double labeling using the marker β-tubulin for neurons, and GFAP for astrocytes, showed that BRUCE was predominantly expressed by neuroblasts (Fig. 1E).

3.2. Downregulation of BRUCE reduces NPC number

To study the function of BRUCE, we employed antisense (AS) oligonucleotides to downregulate its expression in the NPCs. The AS-BRUCE construct lowered the protein levels significantly and decreased the number of viable cells by about 40% (Fig. 2A and B). Downregulation of BRUCE may influence cell death, but the number of cells with fragmented DNA did not change (Fig. 2C), nor was there an increase in the number of cells with activated caspase-3 (data not shown). Staurosporine enhances cell death and decreased cell viability by about 20% in control NPCs. This value was the same in AS-BRUCE treated NPCs (Fig. 2A), indicating that

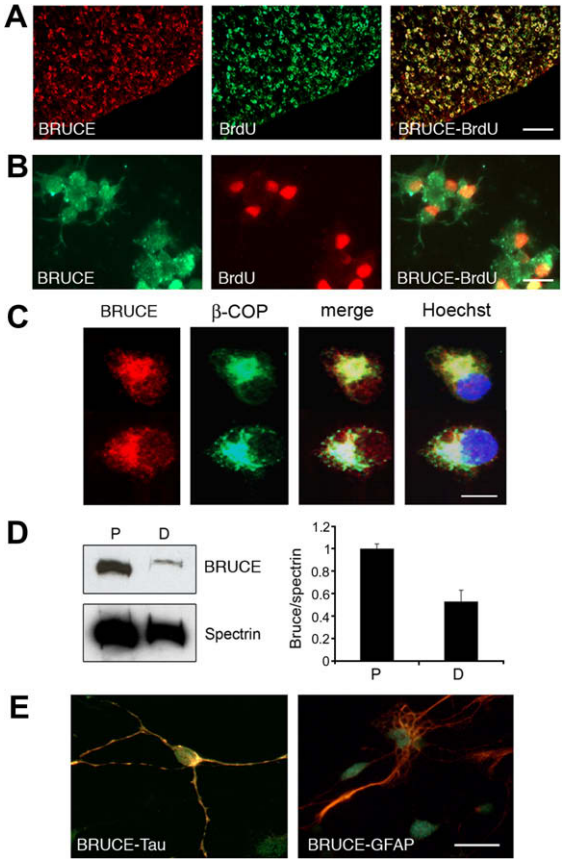

Fig. 1. BRUCE is expressed by dividing NPCs. (A) BrdU was administrated to E17-old rats to label dividing cells and brain sections were immunostained using antibodies against BRUCE (red) and BrdU (green) as described in Section 2. Merge picture shows co-expression in the neuroepithelium (yellow). Size bar, 75 μm. (B) NPCs from E17-old rat brain were incubated as neurospheres and stained for BRUCE (green). BrdU (10 μM) was added for the last 24 h and dividing cells detected by staining (red). Note co-expression of BRUCE and BrdU (yellow) in dividing cells. Size bar, 10 μm. (C) NPCs were dissociated into single cells. Immunostaining showed BRUCE (red) co-lozalized (yellow) with the Golgi marker β-COP (green). Nuclei are stained with Hoechst, blue. Size bar, 5 μm. (D) Immunoblots of proliferating (P) and differentiated (D) NPCs. Quantification expressed relative to α-spectrin. (E) Immunostaining of BRUCE (green) in differentiated NPCs in conjunction with markers for neurons (Tau) and astrocytes (GFAP) (red). Note expression in differentiating neuroblasts and less in astrocytes. Size bar, 20 μm.

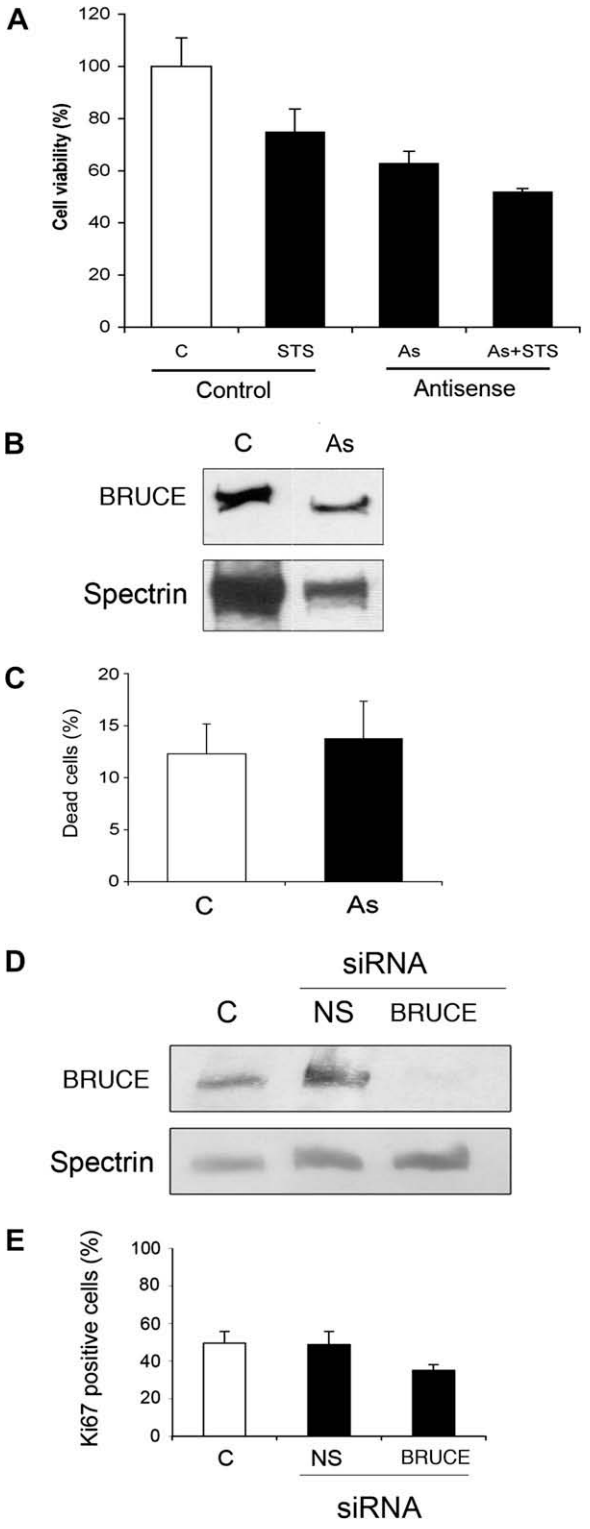

Fig. 2. Downregulation of BRUCE decreases the number of NPCs. BRUCE levels were reduced in NPCs using antisense oligonucleotides (AS-BRUCE) (A and B) or using siRNA (siRNA-BRUCE) (D and E). Cells were cultured for 72 h. In some cultures 20 nM staurosporine (STS) was added for 24 h to induce cell death. (A) Viable cells quantified by the MTT assay. Values are means ± S.E.M., $n = 5$. $P \leqslant 0.001$ for AS vs C; $P \leqslant 0.01$ and for STS vs C. (B) BRUCE levels shown by immunoblots. (C) Cell death studied by counting number of apoptotic nuclei. Values are means ± S.E.M., $n = 5$. No significant difference between groups (D) siRNA treatment reduced BRUCE levels by over 70% compared with control. NS, nonsense, control siRNA. (E) The number of Ki67-positive proliferating cells was decreased in siRNA treated cells. Values are means ± S.E.M., $n = 5$. $P \leqslant 0.005$ for siRNA vs C.

the effect of BRUCE is not related to enhancement of cell death. siRNA against BRUCE reduced protein levels in the NPCs (Fig. 2D), and the number of Ki67-positive cells (Fig. 2E). This shows that the level of BRUCE is important in controlling cell proliferation in NPCs cultivated as neurospheres.

3.3. Dex regulates BRUCE and BRUCE overexpression attenuates the effect of Dex

We have previously observed that Dex negatively influences NPCs by decreasing cell proliferation [3]. Dex also regulates BRUCE in the NPCs by rapidly reducing BRUCE-mRNA levels – and subsequently the protein levels (Fig. 3A and B). This effect was dependent on GH receptors as shown by the inhibitor Mifepristone. In this context the GH receptor was translocated to the cell nuclei (data not shown). Interestingly BRUCE-mRNA levels seem to recover at later time points after Dex although protein levels did not (Fig. 3A), suggesting a dual regulation of BRUCE at both RNA and protein levels. To study the link between BRUCE and Dex, we added Dex to NPCs overexpressing BRUCE. BRUCE expression counteracted the negative effect of Dex on cell proliferation (Fig. 3C). Previously, Dex was found to decrease the levels of cyclinD1 in NPCs [3]. To study whether cyclinD1 influences BRUCE we performed siRNA experiments, which showed that the decrease in cyclinD1 did not influence BRUCE levels (Fig. 3D).

3.4. Usp8 is increased by Dex in the NPCs

In human embryonic kidney cells the levels of BRUCE is controlled by the ubiquitin ligase, Nrdp1 [17]. However, the deubiquitinating enzyme Usp8/Ubpy affects the stability of Nrdp1 [18], and thereby may influence BRUCE. We observed that the proteasome inhibitor MG132 elevated BRUCE in NPCs, showing that the protein

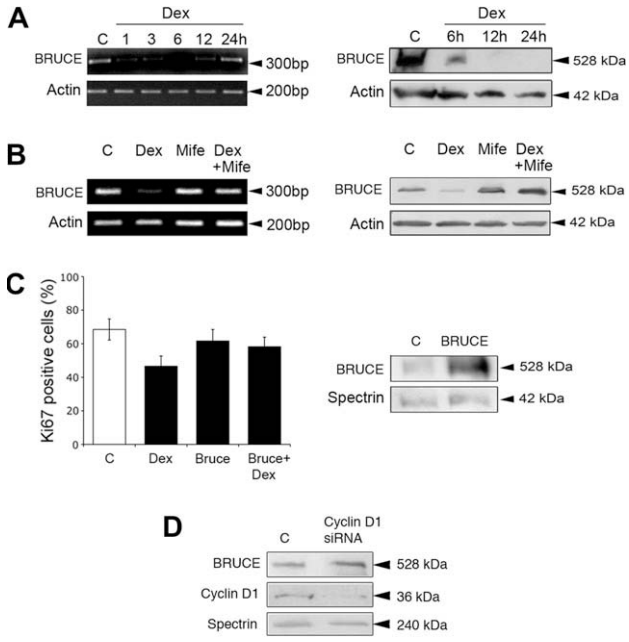

Fig. 3. Dexamethasone decreases BRUCE in NPCs. NPCs were incubated with 1 μM dexamethasone (Dex) for different times. Two microns of Mifepristone (Mife) was added as indicated. (A) Left panel, semiquantitative PCR was done as described in Section 2; right panel, immunoblot. Note sustained decrease in BRUCE protein at 24 h. (B) Left panel, PCR. Right panel, immunoblot. Note inhibition of the Dex effect by Mife. (C) BRUCE was overexpressed in NPCs and the cells were further treated with Dex for 24 h. BRUCE counteracts Dex-induced inhibition of cell proliferation as shown by Ki67 labeling. (D) CyclinD1 was downregulated by silencing RNA. No influence on BRUCE levels in siRNA treated cells.

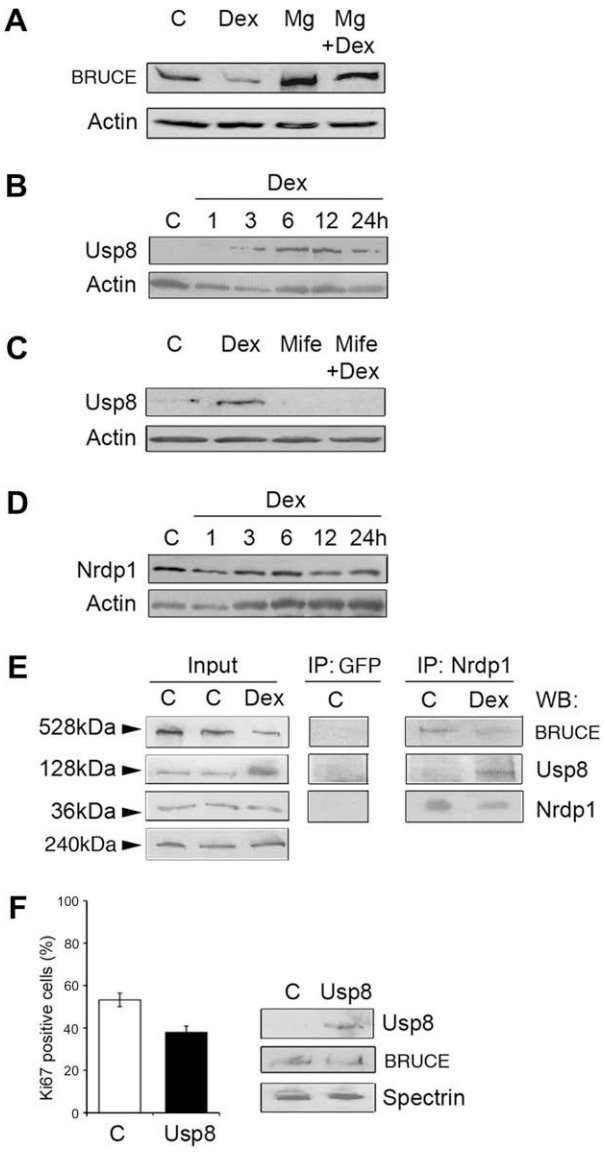

Fig. 4. Effects of Dex on Usp8 and Usp8-Nrdp1 interaction. Cells were incubated with 1 μM Dex for different time periods. (A) Immunoblot. Cells were treated with Dex for 6 h in the absence or presence of 3 μM MG132 (Mg) to inhibit proteasomes. Note increase in BRUCE. (B) Immunoblot. Dex increases Usp8 levels after 3–6 h. (C) Cells were incubated for 6 h with Dex in the presence or absence of 2 μM Mife. (D) Cells were incubated for 6 h. Nrdp1 level not affected by Dex. (E) Cells were incubated with Dex for 6 h. Immunoprecipitation using anti-Nrdp1 antibodies followed by immunoblotting was done as described in Methods. Note interaction of Usp8, BRUCE and Nrdp1 in Dex treated cells. Control immunoprecipitations were done using unspecific IgGs. (F) Usp8 was overexpressed in NPCs for 24 h and cells were analyzed for cell proliferation using Ki67 labeling. Immunoblots show elevated Usp8 and a decrease in BRUCE.

undergoes proteasomal degradation (Fig. 4A). Nrdp1 and Usp8 are both expressed by the NPCs (Fig. 4B and D). Treatment with Dex upregulated the levels of Usp8 in the NPCs, and this effect was blocked by Mifepristone (Fig. 4B and C). The levels of Nrdp1 were not significantly increased by Dex (Fig. 4D). However, Usp8 and Bruce were co-immunoprecipitated with Nrdp1, particularly in hormone-treated cells, showing a possibility for a functional interaction in the NPCs (Fig. 4E). To study the coupling between Usp8 and Bruce in more detail, we overexpressed Usp8 in the NPCs, which led to decreased levels of Bruce and a reduced number of proliferating cells (Fig. 4F).

4. Discussion

The IAPs have an established function in cell death control [4,5]. Apart from cell death, some IAPs members such as survivin affect cell division [5]. Recent studies in yeast, insects and in nematode have identified IAP homologues that influence the segregation of chromosomes and play a role in cytokinesis. These proteins bear baculoviral inhibition of apoptosis protein repeat (BIR) domains that are partly distinct from the mammalian IAPs. The whole protein family is then best characterized as BIR containing proteins (BIRC proteins).

The IAPs contain a motif of about 70 amino acids, called the BIR domain that binds caspases, and some also carry a RING finger domain involved in protein ubiquitination [4,5]. BRUCE/Apollon or BIRC6 is a large protein that carries a BIR domain in the aminoterminal and an ubiquitin conjugation motif in the carboxyterminal region [6]. BRUCE may have a dual function in cells and influence cell death and ubiquitination processes [7–10]. It was reported that BRUCE gene null mice exhibit increased apoptosis. However, other studies showed no signs of enhanced cell death [9,10]. The decrease in the number of placental spongiotrophoblasts observed may be secondary to a reduced cell proliferation or insufficient differentiation [9,10].

We show here that BRUCE is expressed by NPCs in developing neuroepithelium and in culture. BRUCE was expressed predominantly in proliferating NPCs, and localized to nuclei in dividing cells in neurospheres and mainly to intracellular membranes including the Golgi in dissociated ones (Fig. 1B and C). Apart from NPCs, BRUCE was present in neuroblasts, which is in line with our previous observations on hippocampal neurons [12]. To study the function of BRUCE in NPCs, we used either AS oligonucleotides or specific siRNAs. Both methods led to a significant downregulation of BRUCE and also reduced the number of NPCs in culture with no signs of increased cell death. Recent studies have shown that BRUCE influences cytokinesis and particularly the formation of the midbody ring that is crucial for cell cycle continuation [11]. In this process BRUCE may act by ubiquitinylating different protein targets to ensure proper cell division.

In view of the role of BRUCE in cell division, it is crucial to know how the protein itself is regulated. We observed that Dex decreases BRUCE expression in NPCs in a GH receptor dependent manner. In comparison with the positive regulation of target genes by GH, less is known about events mediating negative effects of the hormones [19]. The rapid downregulation of BRUCE-mRNA may occur through a transcriptional silencing or be due to an effect on mRNA stability. The promoter region of *BRUCE* has so far not been studied and will require more analysis. Apart from a direct effect on gene expression, Dex may influence BRUCE through the recently described Usp8-Nrdp1 system [17,18]. Nrdp1 was reported to decrease BRUCE in human tumor cells and increase cell death [17]. Moreover, Nrdp1 itself is influenced by Usp8, which stabilizes the protein [18]. We observed that Dex elevated Usp8 in the NPCs through an involvement of GH receptors. This upregulation of Usp8 by Dex was slower than the negative effect observed for BRUCE-mRNA after Dex. In our experiments, Dex did not significantly upregulate Nrdp1 as shown in immunoblots (Fig. 4). However, as shown in co-immunoprecipitation experiments Nrdp1 interacts with both Usp8 and BRUCE particularly in hormone-treated cells, suggesting a functional coupling. Moreover, in overexpression studies Usp8 readily decreased BRUCE, showing that the increased Usp8 activity can regulate BRUCE either directly or indirectly. It is likely that the increases observed in Usp8 after Dex contributes to the sustained decrease in BRUCE protein at later time points in hormone-treated NPCs when the BRUCE-mRNA levels are recovering (Fig. 2A). In line with this notion, expression of Usp8 in the NPCs reduced BRUCE levels and decreased cell proliferation.

Taken together the results show that the cellular levels of BRUCE are crucial in controlling cell division in NPCs, and possibly in other stem cell populations. The downregulation of BRUCE in NPCs did not significantly increase cell death, but it negatively influenced NPC proliferation. The regulation of BRUCE by Dex involved GH receptors and may occur by repression of *BRUCE* gene expression in conjunction with reduced protein stability secondary to increases in Usp8 levels by Dex. The precise molecular mechanism underlying the GH-mediated effects of BRUCE on NPC proliferation and its physiological consequences for neurodevelopment warrant further studies.

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References

[1] McKay, R. (1997) Stem cells in the central nervous system. Science 276, 66–71.
[2] Gage, F.H. (2000) Mammalian neural stem cells. Science 287, 1433–1438.
[3] Sundberg, M., Savola, S., Hienola, A., Korhonen, L. and Lindholm, D. (2006) Glucocorticoid hormones decrease proliferation of embryonic neural stem cells through ubiquitin-mediated degradation of cyclin D1. J. Neurosci. 26, 5402–5410.
[4] Hauser, H.P., Bardroff, M., Pyrowolakis, G. and Jentsch, S. (1998) A giant ubiquitin-conjugating enzyme related to IAP apoptosis inhibitors. J. Cell Biol. 14, 1415–1422.
[5] Hao, Y., Sekine, K., Kawabata, A., Nakamura, H., Ishioka, T., Ohata, H., Katayama, R., Hashimoto, C., Zhang, X., Noda, T., Tsuruo, T. and Naito, M. (2004) Apollon ubiquitinates SMAC and caspase-9, and has an essential cytoprotection function. Nat. Cell Biol. 6, 849–860.
[6] Ren, J., Shi, M., Liu, R., Yang, Q.H., Johnson, T., Skarnes, W.C. and Du, C. (2005) The Birc6 (Bruce) gene regulates p53 and the mitochondrial pathway of apoptosis and is essential for mouse embryonic development. Proc. Natl. Acad. Sci. USA 102, 565–570.
[7] Salvesen, G.S. and Duckett, C.S. (2002) IAP proteins: blocking the road to death's door. Nat. Rev. Mol. Cell Biol. 3, 401–410.
[8] Vaux, D.L. and Silke, J. (2005) IAPs, RINGs and ubiquitylation. Nat. Rev. Mol. Cell Biol. 6, 287–297.
[9] Lotz, K., Pyrowolakis, G. and Jentsch, S. (2004) BRUCE, a giant E2/E3 ubiquitin ligase and inhibitor of apoptosis protein of the trans-Golgi network, is required for normal placenta development and mouse survival. Mol. Cell Biol. 24, 9339–9350.
[10] Hitz, C., Vogt-Weisenhorn, D., Ruiz, P., Wurst, W. and Floss, T. (2005) Progressive loss of the spongiotrophoblast layer of Birc6/Bruce mutants results in embryonic lethality. Genesis 42, 91–103.
[11] Pohl, C. and Jentsch, S. (2008) Final stages of cytokinesis and midbody ring formation are controlled by BRUCE. Cell 132, 832–845.
[12] Sokka, A.-L., Mudo, G., Aaltonen, J., Belluardo, N., Lindholm, D. and Korhonen, L. (2005) Bruce/Apollon promotes hippocampal neuron survival and is downregulated by kainic acid. Biochem. Biophys. Res. Commun. 338, 729–735.
[13] Brännvall, K., Korhonen, L. and Lindholm, D. (2002) Estrogen-receptor-dependent regulation of neural stem cell proliferation and differentiation. Mol. Cell Neurosci. 21, 512–520.
[14] Brännvall, K., Bogdanovic, N., Korhonen, L. and Lindholm, D. (2005) 19-Nortestosterone influences neural stem cell proliferation and neurogenesis in the rat brain. Eur. J. Neurosci. 21, 871–878.
[15] Korhonen, L., Hansson, I., Kukkonen, J.P., Brännvall, K., Kobayashi, M., Takamatsu, K. and Lindholm, D. (2005) Hippocalcin protects against caspase-12-induced and age-dependent neuronal degeneration. Mol. Cell Neurosci. 28, 85–95.
[16] Row, P.E., Liu, H., Hayes, S., Welchman, R., Charalabous, P., Hofmann, K., Clague, M.J., Sanderson, C.M. and Urbé, S. (2007) The MIT domain of UBPY constitutes a CHMP binding and endosomal localization signal required for efficient epidermal growth factor receptor degradation. J. Biol. Chem. 282, 30929–30937.
[17] Qiu, X.B., Markant, S.L., Yuan, J. and Goldberg, A.L. (2004) Nrdp1-mediated degradation of the gigantic IAP, BRUCE, is a novel pathway for triggering apoptosis. EMBO J. 23, 800–810.
[18] Wu, X., Yen, L., Irwin, L., Sweeney, C. and Carraway 3rd, K.L. (2004) Stabilization of the E3 ubiquitin ligase Nrdp1 by the deubiquitinating enzyme USP8. Mol. Cell Biol. 24, 7748–7757.
[19] Dostert, A. and Heinzel, T. (2004) Negative glucocorticoid receptor response elements and their role in glucocorticoid action. Curr. Pharm. Des. 10, 2807–2816.